

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

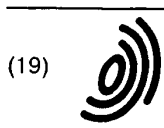
Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) **EP 1 055 727 A2**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication: 29.11.2000 Bulletin 2000/48
(51) Int. Cl.⁷: **C12N 15/12**, C12N 9/10, C12N 15/11, A01H 5/00
(21) Application number: 00304522.6
(22) Date of filing: 26.05.2000

(84) Designated Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
Designated Extension States:
AL LT LV MK RO SI

(30) Priority: 26.05.1999 JP 14635899

(71) Applicant:
mitsui chemicals, inc.
Tokyo (JP)

(72) Inventors:
• Mizuno, Misako
Ushiku-shi, Ibaraki (JP)

• Ashihara, Hiroshi
Tokyo (JP)
• Mizuno, Kouichi
Ushika-shi, Ibaraki (JP)
• Fujimura, Tatsuhito
Tsukuba-shi, Ibaraki (JP)

(74) Representative:
Harvey, David Gareth et al
Graham Watt & Co.
Riverhead
Sevenoaks Kent TN13 2BN (GB)

(54) **Cloning of an N-methyltransferase involved in caffeine biosynthesis**

(57) Thanks to the present invention, N-methyl transferase that can be utilized as an industrial, food, or medical enzyme, can be produced efficiently. The present invention makes it possible to modify caffeine biosynthesis metabolism of caffeine productive plants, plant tissues, or plant cells, for efficiently producing caffeine metabolism based compounds. Furthermore, the caffeine biosynthesis metabolism of caffeine productive plants, plant tissues, or plant cells can be modified, thereby modifying the production rate of a caffeine metabolism based compound group.

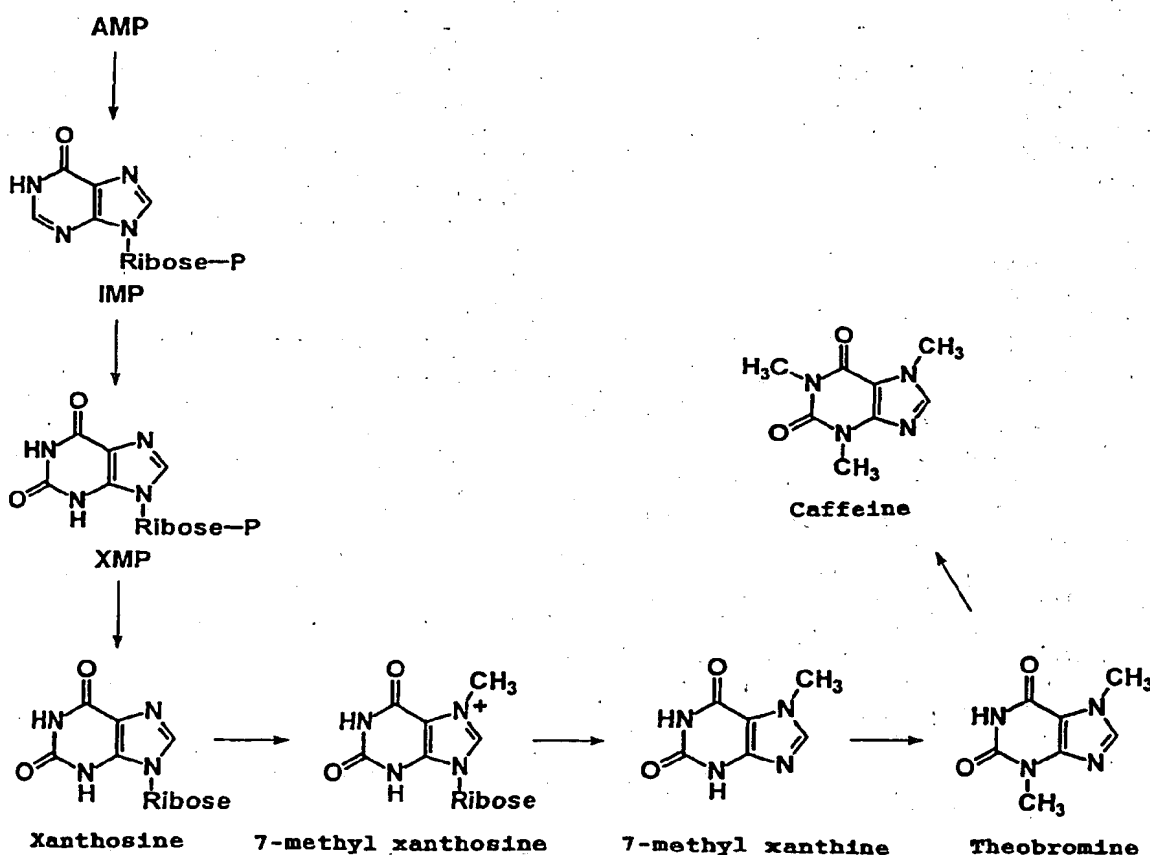
EP 1 055 727 A2

Description

[0001] The present invention relates to a N-methyl transferase, one of the enzymes constituting a caffeine synthesis system, which is a polypeptide simultaneously having activities of three methyl transferases, 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase and paraxanthine N3 methyl transferase, and variants of the N-methyl transferase; DNA molecules or RNA molecules having nucleotide sequences encoding any one of the N-methyl transferase and the variants thereof; vectors using these molecules; and cells transformed with the vectors and their uses.

[0002] Caffeine is a purine alkaloid contained in Theaceae Camellia plants such as Camellia sinensis or Rubiaceae Coffea plants such as Coffea arabica or the like, and is used as a raw material for medicines or a food additive. At present, caffeine is produced by extraction from caffeine productive plants including the foregoing plant species or organic synthesis. In addition, in gusts such as tea or coffee, in order to alleviate or enhance their stimulus, a reduction or increase in the content of caffeine and its intermediates is attempted using classical breeding techniques or the like.

[0003] In Phytochemistry, 31, 2575- (1992), there is disclosed by experiments using ^{14}C -tracer that caffeine is biosynthesized from xanthosine through three stages of N-methylation. This reaction path is shown below.



[0004] Enzyme activities for catalyzing this methylation, that is, methyl transferase activities, were first reported by a study using coarse extraction of tea leaves in 1975 (Biochem. J. 146, 87- (1975)). Although attempts have been made to purify methyl transferase from coffee (Phytochemistry, 37, 1577- (1994)), the purification magnification is very low. For tea, partial purification of methyl transferase was reported (Physiol. Plant., 98, 629- (1996)), but no enzyme protein was isolated.

[0005] As described above, concerning the amino acid sequence of the enzyme in a caffeine synthesis system, that is, a N-methyl transferase that catalyzes a two-stage methylation reaction from 7-methyl xanthine through theobromine to caffeine that is a final reaction of caffeine biosynthesis, neither amino acid sequence nor DNA encoding the amino

acid sequence is known in the prior art.

[0006] It is an object of the present invention to provide N-methyl transferase, one of the enzymes that constitute a caffeine synthesis system useful for caffeine synthesis, simultaneously having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase, and paraxanthine N3 methyl transferase, a DNA or RNA molecule encoding the N-methyl transferase useful for enhancement or suppression of caffeine production in microor-

[0007] For example, all or part of the DNA molecule according to the present invention is incorporated in microorganisms or plant cells in a form of sense or antisense, thereby making it possible to achieve the following objects:

- (1) Efficiently producing N-methyl transferase that can be utilized as an enzyme for industrial, food, or medical use;
- (2) Efficiently producing compounds related to caffeine metabolism by modifying caffeine biosynthesis and metabolism of caffeine productive plants, plant tissues or plant cells; and
- (3) Modifying caffeine biosynthesis and metabolism of caffeine productive plants, plant tissues, or plant cells, thereby modifying the production rate of the caffeine metabolism related compounds.

[0008] The present inventors conducted N-terminal amino acid sequence analysis of a N-methyl transferase, as a polypeptide simultaneously having activities of three enzymes, 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase and paraxanthine N3 methyl transferase, that has been purified from cotyledons of tea, as a result of the inventor's earnest study. Based on the result, a DNA probe was prepared, and a target DNA molecule were successfully isolated by RT-PCR techniques and 5' RACE techniques using this probe.

[0009] Next, the DNA molecule was integrated into a vector and inserted into *Escherichia coli* to express a large amount of a polypeptide derived from the DNA molecule. When the expressed polypeptide was recovered and its enzymological properties were investigated, the same reaction as that of the polypeptide having activities of the above described three N-methyl transferases isolated from cotyledons of tea, that is, caffeine production from paraxanthine was observed. It was thus verified that the DNA molecule has a gene that encodes a N-methyl transferase, one of the enzymes constituting a caffeine synthesis system, simultaneously having activities of the above described three N-methyl transferase.

[0010] In plants in which N-methylation is carried out for a xanthosine or analogous compound, or a xanthine or analogous compound, using S-adenosyl methionine (SAM) as a methyl-group donor, polypeptides having the activity of the N-methyl transferase according to the present invention or the same enzyme activity as the transferase and DNAs that encode these peptides are estimated to be contained. Using the method according to the present invention, the N-methyl transferase or the substantially same enzyme as the transferase and DNA or RNA molecules that encode these transferases can be obtained from these plants.

[0011] The present inventors achieved the present invention based on the above findings. That is, the present invention includes the following aspects.

[0012] The first DNA molecule according to the present invention are characterized by comprising any one of the following nucleotide sequences:

- (a) a nucleotide sequence that encodes a N-methyl transferase, as a polypeptide having an amino acid sequence of SEQ ID NO: 1 of the sequence listing and having enzyme activities of 7 methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase and paraxanthine N3 methyl transferase; and
- (b) a modified nucleotide sequence obtained by nucleotide replacement, deletion or insertion in the above described nucleotide sequence (a) within a range where a polypeptide encoded by the nucleotide sequence (a) can maintain the enzyme activity.

[0013] It is preferable that the modified nucleotide sequence (b) can be hybridized with the nucleotide sequence (a) under stringent conditions.

[0014] The first RNA molecule according to the present invention are characterized by comprising any one of the following nucleotide sequences:

- (a) a nucleotide sequence that encodes the N-methyl transferase, a polypeptide having an amino acid sequence of SEQ ID NO: 1 of the sequence listing and having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase and paraxanthine N3 methyl transferase; and
- (b) a modified nucleotide sequence obtained by nucleotide replacement, deletion or insertion in the above described nucleotide sequence (a) within a range where a polypeptide encoded by the nucleotide sequence (a) can maintain the enzyme activities.

[0015] It is preferable that the modified nucleotide sequence (b) can be hybridized with the nucleotide sequence (a)

under stringent conditions.

[0016] A vector for expression of the N-methyl transferase according to the present invention is characterized by comprising the above described DNA molecule and a constitution to express the N-methyl transferase encoded by the first DNA molecule, in plant cells. Host cells can be transformed using the expression vector to obtain transformed cells. Further, the transformed cells are cultured, thereby making it possible to produce N-methyl transferase having the above enzyme activities.

[0017] Another aspect of a DNA molecule according to the present invention is the second DNA molecule having a sequence complementary to all or a part of the nucleotide sequence of the above first DNA molecule, characterized by that the second DNA molecule is capable of inhibiting the enzyme activities of the plant cells when it is introduced into plant cells having the enzyme activities and expressed in the plant cells.

[0018] Another aspect of a RNA molecule according to the present invention is the second RNA molecule having a sequence complementary to all or a part of the nucleotide sequence of the above first RNA molecule, characterized by that the second RNA molecule is capable of inhibiting expression of the N-methyl transferase activities of the plant cells when it is introduced into plant cells having the N-methyl transferase activities and its expression is conducted.

[0019] An aspect of a vector according to the present invention is characterized by comprising any of the above DNA molecules and RNA molecules. This vector can be provided so as to have a function either to make expression of the N-methyl transferase possible or to inhibit expression of the N-methyl transferase in a microorganism or a plant, which transferase has the three activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase, and paraxanthine N3 methyl transferase as described above.

[0020] Using the vector, microorganisms, plant cells, plant tissues, or plant bodies can be transformed, and the obtained transformants are included in the present invention. A secondary metabolite in plants can be produced using the transformed plant cells, plant tissues, or plant bodies. In addition, the composition of the secondary metabolite in the transformed plants can be modified using the plant cells, plant tissues, or plant bodies.

[0021] The N-methyl transferase according to the present invention is a polypeptide having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase and paraxanthine N3 methyl transferase, characterized by having:

(a) an amino sequence of SEQ ID NO: 1 of the sequence listing; or

(b) a modified amino acid sequence obtained by replacement, insertion, or deletion of amino acids for the amino acid sequence of SEQ ID NO: 1 of the sequence listing within a range where the above described enzyme activities are not lost, but maintained.

[0022] As this modified amino acid sequence (b), it is preferable that the DNA encoding the amino acid sequence (a) and DNA encoding this modified amino acid sequence (b) can be hybridized under stringent conditions.

[0023] According to the present invention, there is provided a DNA molecule and a RNA molecule encoding the N-methyl transferase, which is:

i) one of the enzymes constituting a caffeine synthesis system,

ii) useful for caffeine synthesis and modification of the composition of caffeine produced in microorganisms or plants, and

iii) a polypeptide simultaneously having the three enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase, and paraxanthine N3 methyl transferase.

Detailed Description of the Invention and Preferred Embodiments

[0024] The N-methyl transferase according to the present invention is a polypeptide simultaneously having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase and paraxanthine N3 methyl transferase.

[0025] As for the N-methyl transferase, that having an amino acid sequence indicated by SEQ ID NO: 1 and that having a modified amino acid sequence obtained by carrying out amino acid replacement, insertion, or deletion within a range where desired N-methyl transferase activity is not damaged in the amino acid sequence of SEQ ID NO: 1 can be mentioned. That is, polypeptides having an amino acid sequence of SEQ ID NO: 1 having the desired N-methyl transferase activity as described above and those having a modified sequence are referred to as N-methyl transferase.

[0026] The above polypeptide having the modified amino acid sequence itself has functions substantially identical to N-methyl transferase of cotyledons of tea, and has high homology with an amino acid sequence of SEQ ID NO: 1 at a site associated with enzyme activities.

[0027] In general, between a plurality of enzymes having the identical functions, it is well known that homology of the amino acid sequence other than a site indispensable for enzyme activities is very low (Kawagoe et al., Proc. Natl.

Acad. Sci. USA, 93, 12082- (1996)). Therefore, even in the case where the entire homology is low, transferase having high homology at a site associated with activities can be classified as N-methyl transferase.

[0028] In comparing the entire amino acid sequence, there can be exemplified a modified amino acid sequence which can provide a polypeptide having the desired N-methyl transferase and homology of 15% or more, preferably homology of 30% or more, more preferably homology of 45% or more, and further preferably homology of 60% or more, and still further preferably homology of 75% or more, the most preferably homology of 95% or more against the amino acid sequence of SEQ ID NO: 1.

[0029] In the case where modification against the amino acid sequence of SEQ ID NO: 1 as the basis are defined at a level of nucleotide sequence encoding the modified amino acid sequence, there can be provided a modified nucleotide sequence having homology of 40% or more, preferably homology of 60% or more, more preferably homology of 75% or more, further preferably homology of 90% or more, still further preferably homology of 95% or more against the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1.

[0030] A nucleotide sequence encoding the N-methyl transferase according to the present invention, that is, an N-methyl transferase gene can contain a nucleotide sequence encoding an amino acid sequence of SEQ ID NO: 1. A specific example thereof can include DNA sequence of SEQ ID NO: 2 and RNA sequence of SEQ ID NO: 3. A nucleotide sequence having homology ruled above against the N-methyl transferase gene as the basis is also included in the gene encoding the N-methyl transferase according to the present invention.

[0031] As a modified amino acid sequence maintaining the desired N-methyl transferase activities, its preferable examples include that encoded by a modified nucleotide sequence, which can hybridize under stringent conditions with the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1 as the basis.

[0032] In addition, as the modified N-methyl transferase gene, that capable of hybridizing with the nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 1 under stringent conditions can be preferably utilized in practice. A specific example thereof can include DNA molecules capable of being hybridized under stringent conditions for a nucleotide sequence of SEQ ID NO: 2; and RNA molecules capable of being hybridized under stringent conditions for a nucleotide sequence of SEQ ID NO: 3.

[0033] Hybridization under these stringent conditions can be carried out, for example, by the method described in Molecular Cloning: Cold Spring Harbor Laboratory Press, Current Protocols in Molecular Biology; Wiley Interscience. As a commercially available system, a GenelImage system (Amasham) can be exemplified. Specifically, hybridization can be carried out by the following operation.

[0034] A membrane to which DNA or RNA molecules to be tested has been transferred is treated for hybridization using a labeled probe in a hybridization buffer specified by the protocol in accordance with product protocols. The composition of hybridization buffer consists of 0.1 wt.% SDS; 5 wt.% Dextran sulfate; 1/20 volume of a blocking reagent included in the kit and 2 to 7 × SSC. A blocking reagent is used by preparing 100 × Denhardt's solution, 2% (weight/volume) Bovine serum albumin, 2% (weight/volume) Ficoll™ 400, 2% (weight/volume) polyvinyl pyrrolidone at a 5-fold concentration, and diluting them to 1/20. 20 × SSC is a 3M sodium chloride and 0.3M citric acid solution. SSC is preferably used at a concentration of 3 to 6 × SSC, and further preferably used at a concentration of 4 to 5 × SSC.

[0035] The hybridization temperature ranges from 40 to 80°C, more preferably 50 to 70°C, and further preferably 55 to 65°C. Incubation for several hours or one night is carried out, followed by washing using a washing buffer. The washing temperature is preferably equal to room temperature, and more preferably is a temperature during hybridization. The composition of the washing buffer is a 6 × SSC + 0.1 wt.% SDS solution, more preferably 4 × SSC + 0.1 wt.% SDS solution, further preferably 2 × SSC + 0.1 wt.% SDS solution, still further preferably 1 × SSC + 0.1 wt.% SDS solution, and the most preferably 0.1 × SSC + 0.1 wt.% SDS solution. A membrane is washed by such washing buffer, whereby the DNA molecules or RNA molecules in which the probe is hybridized can be identified by utilizing a label employed for the probe.

[0036] Modification may occur in nature or may be artificially generated by site mutation in the nucleotide sequence.

[0037] DNA molecules having N-methyl transferase genes according to the present invention can be separated from cells producing N-methyl transferase according to the present invention by utilizing PCR technique as described in "Plant PCR test protocols" (another volume of cell engineering, plant cell engineering series 2) Shujynsha (1995), in which an oligo-nucleotide specifically hybridized for DNA molecules for encoding N-methyl transferase, for example, is employed as a primer.

[0038] Specifically, a linker is bonded with cDNA synthesized from mRNA, and PCR is carried out between the linker and the DNA encoding an amino acid sequence constituting the N-methyl transferase, whereby the full length sequence of the target cDNA can be isolated.

[0039] DNA molecules encoding the N-methyl transferase obtained by such hybridization technique or PCR technique have homology with the N-methyl transferase gene of SEQ ID NO: 2 at least at a site used for isolation. The homology used herein denotes homology of 15% or more, preferably homology of 30% or more, more preferably homology of 45% or more, further preferably homology of 60% or more, still further preferably homology of 75% or more, still furthermore homology of 90% or more, and the most preferably homology of 95% or more in comparing the amino acid

sequence encoded by the respective N-methyl transferase genes. However, even if homology with N-methyl transferase becomes 15% or less as a result of deletion, addition, and replacement of a plurality of residues of amino acid to be encoded, it is estimated that some of the obtained N-methyl transferase genes maintain a region indispensable to the functions of N-methyl transferase, and substantially encode proteins having their functions similar to N-methyl transferase.

[0040] All of the organisms employed for isolating DNA molecules or RNA molecules having nucleotide sequences (genes) encoding N-methyl transferase according to the present invention can be used as long as they produce caffeine or its precursor. They can include Theaceae Camellia plant such as tea, Rubiaceae Coffea plant such as coffee, Sterculiaceae Cola plant such as Cola or the like.

[0041] A RNA molecule including a N-methyl transferase gene according to the present invention can be obtained by connecting a DNA encoding the N-methyl transferase, which can be prepared by the above described method, in the desired direction at the operable location on the downstream side of a promoter such as Sp6 promoter or T7 promoter, which is recognized by RNA polymerase, to prepare a recombinant molecule and translating the recombinant molecule by Sp6 RNA polymerase or T7 polymerase to obtain the desired RNA molecule. The RNA molecule can be also obtained by introducing a DNA or RNA encoding the N-methyl transferase into a plant virus or inserting a DNA or RNA encoding the N-methyl transferase into a vector carrying an appropriate expression cassette as described below so that the DNA or RNA is connected to the expression cassette in the desired direction at the operable location to prepare a recombinant molecule and introducing the recombinant molecule into a host microorganism or plant so that the RNA encoding the N-methyl transferase can be formed in the host using the transcription activity of the host.

[0042] DNA molecules having complementarity with all or a part of the DNA molecules having the N-methyl transferase genes or RNA molecules having complementarity with all or a part of the RNA molecules having N-methyl transferase genes can be used as DNA or RNA molecules for inhibition or suppression of N-methyl transferase expression in plant cells as long as these molecules have a function to inhibit expression of the N-methyl transferase peculiar to the host plant cells when these molecules are expressed in the host plant cells.

[0043] According to the present invention, the part of the N-methyl transferase gene as the basis for the complementary DNA or RNA molecule denotes a site which can be used to provide a complementary sequence as a basis to form mRNA for inhibition (i. e., antisense RNA) in the host cells. This mRNA for inhibition is formed in host cells, the RNA is bonded with mRNA for expressing N-methyl transferase in host cells, and the expression of N-methyl transferase in the host cells is inhibited. This site is necessary for forming such antisense mRNA, which has, for example, at least 14 base length.

[0044] DNA molecules for forming the antisense mRNA can include, for example, DNA molecules complementary to all or a part of the nucleotide sequence SEQ ID NO: 2, and antisense RNA molecules include RNA molecules complementary to all or a part of the nucleotide sequence of SEQ ID NO: 3. DNA molecules or RNA molecules having complementarity with all or a part of the modified sequence capable of being hybridized with these nucleotide sequence of SEQ ID NOs: 2 and 3 under stringent conditions can also be used for such purpose.

[0045] The DNA molecules having high homology with these inhibiting DNA molecules or RNA molecules and having desired inhibiting or restricting functions can also be utilized. Here, high homology denotes homology of 60% or more, preferably homology of 75% or more, further preferably homology of 90% or more, and the most preferably homology of 95% or more in comparing the respective nucleotide sequences.

[0046] These inhibiting DNA molecules or RNA molecules themselves may not always encode N-methyl transferase according to the present invention.

[0047] Another aspect of the nucleotide sequence for inhibiting N-methyl transferase according to the present invention is directed to a nucleotide sequence having one or more sites with homology to the N-methyl transferase gene, but do not encode N-methyl transferase. The N-methyl transferase peculiar to the host cells can be inhibited by replacing this nucleotide sequence with the N-methyl transferase gene of the host cells to be deleted.

[0048] With respect to expression of the N-methyl transferase gene or expression of the DNA molecules having functions for inhibiting or restricting expression of the N-methyl transferase gene or N-methyl transferase in the host cells, an example utilizing plant cells as host cells will be described hereinafter.

[0049] For expression in plant cells, there can be utilized a method for introducing into host plant cells an expression cassette and transforming the host cells, which expression cassette includes: (i) a promoter enabling transcription from DNA to mRNA in the host cell; (ii) a DNA fragment containing a N-methyl transferase gene bonded with the downstream side of the promoter in the sense or antisense direction or a DNA fragment having functions for inhibiting the expression of N-methyl transferase; and (iii) a terminator sequence containing a polyadenylation site required for stabilization of transcribed products bonded with the downstream side of these DNA fragments as required.

[0050] Such expression cassette and a vector containing this cassette are the subject of the present invention.

[0051] The expression cassette can contain a promoter for constitutively or inductively expressing the inserted DNA. In addition, this expression cassette can have a replication origin for its replication in the plant cells as required.

[0052] Promoters for constitutive expression include, for example, a 35S promoter for cauliflower mosaic virus, rice

actin promoter and the like. In addition, promoters for inductive expression include, for example, promoters known to be expressed by external factors such as infection or invasion of mold, bacteria or virus, low temperature, high temperature, dry, anaerobic conditions, specific compound spraying or the like. Such promoters include a promoter of chitinase genes of rice expressed, for example, by infection or invasion of mold, bacteria or virus; a promoter of PR protein genes; a promoter of "lip19" genes of rice induced by low temperature; a promoter of "HSP182" genes of *Capsella bursa-pastoris* induced by high temperature; a promoter of "rab" genes of rice induced by dry; a promoter of alcohol dehydrogenase gene of corn induced by anaerobic conditions or the like. In addition, the promoter for the chitinase genes of rice and the promoter for PR protein genes of tobacco are induced by specific compounds such as salicylic acid or the like, and the rice "rab" gene promoter is induced by spraying abscisic acid of plant hormone.

10 [0053] Alternatively, as a promoter for expressing DNA inserted into the expression cassette, a promoter derived from any of the N-methyl transferase genes per se can be used.

[0054] A specific example of promoter isolation can include a method for selecting genome DNA fragments and specifying the DNA at the upward section of the gene by utilizing the hybridization technique in which all or a part of N-methyl transferase genes is employed as a probe.

15 [0055] In order to prepare for introduction of recombinant DNA molecules in the expression cassette into plants, there can be utilized a number of cloning vectors containing a replication signal in *E. coli* and a marker gene(s) for selection of the transformed *E. coli* cells. Examples of such vectors include pBR322, pUC system, Mi3mp system or the like. A target sequence can be introduced at a proper restriction enzyme cutting site. In order to clarify characteristics of the obtained plasmid DNA, analysis of the restriction enzyme cutting site, gel electrophoresis, and other biochemical, molecule-biological methods are generally used. After each operation has been completed, the plasmid DNA is cut, and can be bonded with another DNA. The sequence of each plasmid DNA can be cloned in the same plasmid or another plasmid.

[0056] In order to introduce the expression cassette into plant cells, a variety of techniques can be used. These techniques include transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation factors; direct induction into protoplast (injection method, electroporation method or the like), Particle gun method or the like and any other possibility.

[0057] In direct introduction into protoplast, no vector is particularly required. For example, a simple plasmid such as pUC derivative can be employed. Some methods for introducing target genes into plant cells may require another DNA sequence. For example, when Ti or Ri plasmid is employed for transformation of plant cells, it is preferable to connect at least right-end sequence of the T-DNA region for the Ti and Ri plasmids, mostly the sequences on both ends so as to be adjacent regions of genes to be introduced.

[0058] When *Agrobacterium* is employed for transformation, an expression cassette to be introduced is required to be cloned in a specific plasmid, that is, in an intermediate vector or a binary vector. The intermediate vector is not replicated in the *Agrobacterium*. The intermediate vector is moved into the *Agrobacterium* by a helper plasmid or an electroporation. The intermediate vector has a region homologous with the sequence of T-DNA, and thus, is incorporated into the Ti or Ri plasmid of *Agrobacterium* by homologous recombination. *Agrobacterium* used as a host is required to include a vir region. In general, the Ti or Ri plasmid includes the vir region, and T-DNA can be moved to plant cells by way of the functions of the region.

40 [0059] On the other hand, the binary vector can be replicated and maintained in *Agrobacterium*. Thus, when the binary vector is incorporated into *Agrobacterium* by the helper plasmid or electroporation technique, T-DNA on the binary vector can be moved to plant cells by the functions of the Vir region of the host.

[0060] The intermediate vector or binary vector including the thus obtained expression cassette and microorganisms such as *Escherichia coli* or *Agrobacterium* including these vectors are the subject of the present invention.

45 [0061] The transformed plant cells can be converted into a plant tissue or, a plant body by experiencing a reproduction process. The reproduction methods depend on the kinds of plant cells, and include the Fujimura et al. method for rice (Plant Tissue Culture Lett., 2, 74- (1995)); the Shillito et al. method for corn (Bio/Technology, 7, 581- (1989)); and the Akama et al. method for *Capsella bursa-pastoris* (Plant Cell Rep., 12, 7- (1992)) or the like.

[0062] According to the present invention, the term "plant body" means the whole individual organism classified into plant or organ parts thereof such as leaves, stems, roots, flowers, fruits, seeds and the likes.

50 [0063] For the plant body produced by these methods or the plant body obtained from its breeding catalyst (for example, seeds, stems, cutting or the like), an expression quantity of N-methyl transferase according to the present invention changes in comparison with a wild type plant body producing caffeine or its precursor; a change in generation quantity of caffeine metabolism system compounds due to modification of metabolism of the host plant or a change in production rate of the caffeine metabolism system compound group due to modification of metabolism of the host plant takes place. The thus-obtained transgenic plant is the subject of the present invention. The plants according to the present invention includes specific tissues or cells of plants such as leaves, flowers, fruits, seeds or the like.

[0064] In addition, in recent years, from study on post-translation gene silencing of plants, it has been found that the expression of target genes can be restricted by utilizing the intrinsic protection mechanisms of the plant for exotic

nucleic acids such as virus (Cell, 95, 177-187 (1998), Chemistry and Biology, 37, 532- (1999) and protein nucleic acid enzyme, 44, 1396- (1999). According to this study, in the case where DNA virus or RNA virus or the like invades plants, the plants transcribe aberrant RNA from these molds, and double-strand RNA is formed sequentially specifically with the transcribed product of the intrinsic sequence that plants possess. This double-strand RNA is decomposed by RNase, thereby making it possible to restrict the expression of target genes (Cell, 96, 303-(1999)). One of the essential characteristics of this method is that a sequence whose expression is to be restricted does not always need to be transformed to the target plant. In addition, further characteristics of this method is that, if a target nucleic acid is introduced into part of the plant (low-order leaf or the like) by infection or the like, its effect widens the entire plant body. A specific expression restricting method is to cause double-strand RNA including all or part of the sequence of the target gene or the sequence having high homology or Agrobacterium having the double-strand DNA to be infected with the low-order leaf of the plant. Here, high homology denotes homology of 60% or more, preferably homology of 75% or more, further preferably homology of 90% or more, and the most preferably homology of 95% or more in comparing the respective base sequences.

[0065] In the plant body subjected to this method, an expression quantity of the N-methyl transferase protein according to the present invention changes in comparison with a wild type plant body producing caffeine or its precursor. In addition, a change in expression quantity of the caffeine metabolism compounds due to modification of metabolism of the host plant or change in production rate of caffeine metabolism system compound group due to modification of metabolism of the host plant takes place. The thus-obtained plant is the subject of the present invention. The plants used in this invention include specific tissues or cells of plants such as leaves, flowers, fruits, seeds or the like.

[0066] Plants producing caffeine with the SAM being a methyl group donor can include caffeine productive plants including Theaceae Camellia plants such as tea; Rubiaceae coffea plants such as coffee; Sterculiaceae Cola plants such as Cola.

[0067] Microorganisms for introducing DNA that encodes the N-methyl transferase, thereby expressing a large amount of the N-methyl transferase proteins according to the present invention can include bacteria such as E. coli, Bacillus subtilis or the like; and virus such as Baculoviridae.

[0068] In addition, any plants producing caffeine or its precursor can be used as plants into which a DNA that encodes the N-methyl transferase according to the present invention in the sense or antisense form to obtain transformed plants for the purposes of improving productivity for a specific compound and changing a production ratio of a specific compound group by modifying the metabolism in host cells.

[0069] The production of secondary metabolites relating to the caffeine synthesis system and the composition of the secondary metabolites produced by these transformants can be modified by culturing plant cells or plant tissues transformed with the vector of the above described constitution or cultivating plants transformed in the same manner. As the secondary metabolites, for example, at least one compound selected from the group consisting of 7-methyl xanthine, paraxanthine, theobromine, and caffeine can be mentioned.

[0070] As supply sources of plant cells, plant tissues, or plant bodies used for transformation, for example, a Camellia plant, a Coffea plant, Cola plant, Ilex plant, Neea plant, Firmiana plant, Paulinia plant, or Therbroma plant body can be mentioned as plant bodies for transformation.

[0071] These plants can include Theaceae Camellia plants such as tea; Rubiaceae coffea plants such as coffee; Sterculiaceae Cola plants such as Cola or the like.

[0072] Further, in the N-methyl transferase according to the present invention, structurally analogous compounds such as 7-methyl xanthine as well as theobromine can be methylated. Thus, even if they are not the above kinds of plants, the method according to the present invention is applicable to plants containing structurally analogous compounds of xanthine thereof.

[0073] As a result of enzymological study, it is clear that the N-methyl transferase according to the present invention has the following basic properties.

[0074] Molecular weight: 41,000 (SDS-PAGE), 61,000 (gel filtration)

Isoelectric point: 4.5 to 5.0 (Chromatofocusing)

Optimum pH: 8.5

Km value: 21 μ M (SAM) or 24 μ M (paraxanthine)

Inhibitor: SAH (S-adenosyl homocysteine)

Reaction mechanism:



Examples

[0075] Hereinafter, the present invention will be described in more detail by way of Examples and Comparative

Examples. The scope of the present invention is not limited to these Examples.

Example 1

5 Preparation of Purified Fraction of N-Methyl Transferase

[0076] First, second, and third leaves of tea leaves (*Camellia sinensis* var. Yabukita) collected in Makurazaki City, Kagoshima Prefecture in May, 1997 were frozen using liquid nitrogen, and preserved at -80°C. This material of 100 g was milled by adding 5 mM EDTA-Na₂ of 1,000 ml; 2.5 mM 2-mercapto-ethanol; 5% (v/v) glycerin; 1 mg aprotinin; 0.5% (w/v) sodium ascorbate; and 50 mM sodium phosphate buffer solution (pH 7.3) containing 2.5% (w/v) insoluble polyvinyl
 10 polypyrrolidone, and was filtrated through three-layer gaze. Then, the filtered liquid was centrifuged (10,000 g, 15 minutes), and a supernatant was obtained. Then, a 50-80% saturated ammonium sulfate fraction was prepared from the supernatant. This fraction was deionized using Sephadex G - 25; was dissolved in 10 mM sodium phosphate buffer solution (pH 7.2) containing 2 mM EDTA-Na₂; 2 mM 2-mercapto-ethanol; 20% (v/v) glycerin; and then, was adsorbed
 15 to hydroxy apatite column (15 × 160 mm) equilibrated with the same buffer solution; and an active fraction was eluted using a 10-200 mM linear concentration gradient of 200 ml of the sodium phosphate buffer solution containing 2 mM EDTA-Na₂; 2mM 2-mercaptoethanol; 20% (v/v) glycerin. The active fraction was collected, precipitation was recollected by 80% unsaturated ammonium sulfate, and the fraction was dissolved in 50 mM Tris - hydrochloric acid buffer solution (pH 8.4) containing 2 mM EDTA-Na₂; 2 mM 2-mercapto-ethanol; 20 mM KCl; 20% (v/v) glycerin. After deionization was
 20 carried out, and the fraction was adsorbed to a cathode ion exchange column Shodex IEC QA-824 (8 × 25 mm) for the exclusive use for high performance liquid chromatography balanced by the same buffer solution. After the column was washed by the same buffer solution, the adsorbed protein was eluted by a linear concentration gradient 30 ml of 20 - 750 mM KCl (dissolved in the 50 mM Tris - hydrochloric acid buffer solution (pH 8.5) containing 2 mM EDTA-Na₂; 2 mM 2-mercapto-ethanol; and 20% (v/v) glycerin). The active fraction was collected, deionized, and adsorbed by adenosine-
 25 agarose (1 ml) that is an affinity column balanced by the same buffer solution after being dissolved in 50 mM Tris - hydrochloric acid buffer solution (pH 8.5) containing 2 mM EDTA-Na₂; 2 mM 2-mercapto-ethanol; and 20% (v/v) glycerin. The active fraction was eluted by the 50 mM Tris - hydrochloric acid buffer solution (pH 8.5) containing 0.2 M NaCl; 2 mM EDTA-Na₂; 2 mM 2-mercapto-ethanol; and 20% (v/v) glycerin. The obtained fraction was subjected to gel filtration using HiLoad Superdex 200 (16 × 600 mm) balanced by the 50 mM Tris - hydrochloric acid buffer solution (pH 8.5) containing 2 mM 2-mercapto-ethanol; 150 mM KCl; 20% (v/v) glycerin, and a finally refined sample was obtained. Table
 30 1 summarizes a change in rate activity in the refining process of N-methyl transferase.

Table 1

Steps	Fraction	Liquid quantity (ml)	Total activity (pkat)	Total protein mass (mg)	Specific activity (pKat/mg)	Degree of Purification	Production rate (%)
1	Crude Extract	930	6330	581	10.9	1.0	100
2	Ammonium sulfate	33.8	3410	155	22.0	2.0	53.9
3	Hydroxy apatite	23.0	2630	28.9	91.0	8.0	41.5
4	Shodex IEC QA-824	7.5	1070	4.82	221	20.3	16.9
5	Adenosine - agarose	2.0	202	0.08	2530	232	3.2
6	Superdex 200	5.8	228	0.04	5700	523	3.6

Example 2

55 **[0077]** Analysis of Amino Acid Sequence of N-Methyl Transferase Refined Fraction

[0078] The finally-purified sample was transferred to a PVDF membrane using a Semidry Blotting apparatus after SDS-polyacrylamide gel electrophoresis. A site at which N-methyl transferase was transferred was cut away, and an amino acid sequence of an N-terminal was analyzed using an ABI protein sequencer. The result is shown in SEQ ID

NO: 4.

Example 3

- 5 **[0079]** 19 residue oligo nucleotide NMT-1 and Not I- (dT) 18 primer (Pharmacia Biotech) based on 7-amino acid residue of the N-terminal of the oligo nucleotide for cDNA cloning of N-methyl transferase was employed as a probe. The sequence of NMT-1 is shown in SEQ ID NO: 5.

Example 4

- 10 **[0080]** Synthesis of a single strand cDNA for cloning of N-methyl transferase gene

(1) Isolation of total RNA

- 15 **[0081]** Young tea leaves of 5 g were milled under the existence of liquid nitrogen using rod or mortar. After sublimation of the liquid nitrogen, 3M-LiCl of 50 ml and 8 M urea were added, and was further milled by polytron. After the milled leaves were statically placed one night at 4°C, and were centrifuged at 12,000 rpm for 15 minutes. Precipitation was suspended in a 0.5% SDS, 10 mM Tris - hydrochloric buffer solution (pH 7.6) to be about 10 ml in total amount. The suspended precipitation was mixed by adding 10 ml phenol chloroform solution, and was centrifuged at 12,000 rpm for 10 minutes. A 1/10 volume of 3M sodium acetate solution (pH 4.8) and then 2 volumes of ethanol were added to the supernatant, the mixture was statically placed at -80°C for one hour. Centrifugation was carried out at 4°C and 12,000 rpm for 10 minutes, 70% ethanol was added to the precipitation separated from the supernatant to form suspension, and centrifugation was carried out again. The supernatant was removed, and the residue was dried up by a vacuum pump. The precipitation thus obtained was dissolved in 1.5 ml water, 150 µl of 3M sodium acetate solution (pH 4.8) was added thereto, and further, 1.5 ml of phenol/chloroform solution was added thereto to be fallen and mixed. Then, centrifugation was carried out at 12,000 rpm for 10 minutes. Ethanol of x2 volume was added to the supernatant, and was statically placed at -80°C for 20 minutes. Then, centrifugation was carried out at 4°C and 12,000 rpm for 10 minutes. To the thus obtained precipitation, 70% ethanol was added, and centrifugation was carried out again. The precipitation was dried up by the vacuum pump, was dissolved in 200 µl water, and thus, a fraction of total RNA was obtained.

(2) Isolation of mRNA

- 35 **[0082]** The total RNA (2 mg) obtained by the above described method was subjected to heat treatment at 65°C for 5 minutes, and then, was mixed with solution A of the same volume of 2-fold concentration (10 mM Tris - hydrochloric acid buffer solution (pH 7.5); 1 mM Na₂ - EDTA; 0.1% SDS; 0.5 M NaCl). 0.1 g of oligo (dT) - Cellulose Type 7 (Pharmacia) was immersed in 2 ml of solution B (10 mM Tris - hydrochloric acid buffer solution (pH 7.5); 1 mM - EDTA-Na₂; 0.1% SDS; 0.1 M NaCl), and the suspension was poured into a blue chip filled with glass wool at its tip end, and was washed by 2.5 ml of 0.1N NaOH. Then, 5 ml of solution A was poured and balanced. Total RNA was applied to this column, and 3 ml of solution A and 4 ml of solution B were poured. Then, mRNA was eluted by 3 ml of solution C (10 mM Tris - hydrochloric buffer solution (pH 7.5), 1 mM - EDTA-Na₂, and 0.05% SDS). The eluted solution was condensed by ethanol precipitation and was dried up. Then, the solution was dissolved in water, and was preserved at -80 °C.

(3) Synthesis of single-strand cDNA

- 45 **[0083]** 190 ng of mRNA was acutely cooled for 3 minutes immediately after heat treatment at 65°C for 10 minutes. This sample was employed as a template, and a single-strand cDNA was synthesized using First-Strand cDNA Synthesis Kit (Pharmacia). The synthesized cDNA was preserved at -20°C.

Example 5

- 50 Cloning of N-Methyl Transferase Genes Using the RT-PCR Technique

- [0084]** The following reaction solution having the single strand cDNA prepared therein by the previously described method was employed as a template was prepared. This reaction solution was reacted at 95°C for 1 minute using Peltier Thermal cycler PTC-200 (Funakoshi). PCR was carried out under conditions in which one cycle included the steps of 95°C/1 minute, 45°C/1 minute, and 72°C/2 minutes, respectively and reacted by 30 cycles, and the reacted product was obtained.

Template cDNA: 3 μ l
 10 \times buffer: 5 μ l
 2.5 mM NTP: 8 μ l
 NMT-1: 1 μ l (50 pmol)
 NotI- (dT) 18: 1 μ l (50 pmol)
 H₂O: 31 μ l
 ExTag (TAKARA): 1 μ l

Example 6

Sub-cloning to Plasmid Vector

[0085] Electrophoresis of the reaction product obtained in Example 5 was carried out in TAE using 0.8% agarose gel, a band of the obtained target product was cut away, and DNA was recollected from a gel using GENE CLEAN (Funakoshi). The recollected DNA was transformed to Escherichia coli DH5 α after ligation with pT7blue vector (Novagen). After color selection was carried out using X-gal, liquid culture was carried out in the LB culture medium containing Ampicillin, and plasmid was extracted and isolated using the alkali-SDS technique. The presence or absence of insert was verified by agarose electrophoresis. Thus, a DNA fragment including the N-methyl transferase gene was isolated into the plasmid.

Example 7

Base Sequence Determination

[0086] Primer extension was carried out in the following reaction solution using isolated plasmid. The reaction conditions are as follows. After reaction at 96 °C/1 minute, PCR in which one cycle included the steps of 96°C/0.2 minute, 50°C/0.1 minute, and 60°C/4 minutes, respectively, and repeated by 25 cycles was carried out. Ethanol precipitation was carried out for the reaction solution, and the obtained DNA was dissolved in Template suppression reagent, and was analyzed using ABI-310 genetic analyzer. In order to determine a sequence at the center of the target DNA, there was used plasmid in which the DNA fragment obtained by treating DNA with Styl was sub-cloned in pUC19. These primer sequences are shown in SEQ ID NOS: 6 and 7.

Composition of the Primer Extension Reaction Solution

[0087]

Plasmid DNA (20 ng): 2 μ l
 Premix: 4 μ l
 Primer: 1 μ l
 H₂O: 3 μ l

Example 8

Isolation of 5' Upstream Area of N-Methyl Transferase mRNA Using 5' the RACE Technique

[0088] 5'- Full RACE Core Set (TAKARA) was employed for isolation of the 5' upstream area. The sequences of the primers used for SEQ ID NOS: 8 to 17 was shown.

[0089] With the 1st strand cDNA synthesized by the method described in Example 4, after decomposition of hybrid RNA and cycling of the single strand cDNA with ligation reaction, PCR reaction was carried out based on normal technique using the primers of SEQ ID NOS: 8 to 12 or SEQ ID NOS: 13 to 17, and the reacted product was obtained. A band of the reacted product was separated by acryl amide electrophoresis, and DNA was recollected from a gel, and was sub-cloned to the pT7blue vector. Then, the base sequence of the DNA inserted by the technique similar to those in Examples 6 and 7 was determined.

Example 9

Expression of N-Methyl Transferase in Escherichia coli

[0090] The following operation was carried out in order to re-incorporate the isolated N-methyl transferase gene into the expression vector pET23d (Novagen).

[0091] PCR was carried out under the following conditions using the pT7blue vector, into which the isolated N-methyl transferase gene DNA obtained in Example 6 as a template and the primers of SEQ ID NOs: 18 and 19, respectively, and the reaction product were obtained.

[0092] Reaction conditions:

After reaction at 95°C/1.5 minutes, PCR in which one cycle included the steps of 95°C/1 minute, 52°C/1 minute, and 72°C/1 minutes, respectively, and repeated by 30 cycles were carried out.

[0092] Separately, a fragment obtained by treating the isolated N-methyl transferase gene DNA fragment with NcoI and EcoRI was inserted into pET23d vector. Then, the above PCR product was further inserted into the NcoI site of this pET23d vector to construct a N-methyl transferase expression plasmid. This plasmid was transformed to Escherichia coli BL21 (DE3). After the obtained Escherichia coli was cultured at 37°C for 2 hours, IPTG was added thereto so as to be 0.3 mM in final concentration, and 3-hour culture was further carried out at 30°C. After culture has been completed, cells were collected, and the cells from 3 ml culture solution was subjected to supersonic treatment for 1 minute with interval in 0.2 ml 10 mM Tris - hydrochloric acid buffer solution (pH 7.5), 0.1 M NaCl, 1 mM EDTA-Na₂. Then, centrifugation was carried out at 14,000 rpm for 10 minutes, and the obtained supernatant was employed as an enzyme solution.

[0093] Regarding the sequencing in Examples 7 and 8, the nucleotide sequence of the DNA fragment including the N-methyl transferase gene obtained in this example has the sequence of SEQ ID NO: 2 and the corresponding RNA nucleotide sequence has the sequence of SEQ ID NO: 3. The corresponding amino acid sequence of the N-methyl transferase is shown as SEQ ID NO: 1.

[0094] The reaction solution for N-methyl transferase measurement was prepared as follows. 10 µl of enzyme solution was added to 100 mM Tris - hydrochloric acid buffer solution (pH 8.5), 0.2 mM MgCl₂, 0.2 mM paraxanthine, 4 µM [methyl-¹⁴C]S - adenosyl methionine (0.9 kBq), and the volume of the reaction solution was 100 µl. Reaction was carried out at 27°C for 10 minutes, the obtained ¹⁴C - caffeine was extracted by adding 1 ml chloroform thereto, and the radiation activity of a chloroform layer was measured. As a control, xanthosine was added to the reaction solution instead of paraxanthine or paraxanthine was removed. As a result of measurement of activity, it was found that 1.56 pmol of caffeine was produced only when paraxanthine was added as a substrate.

[0095] The above enzymatic reaction was repeated except that 7-methyl xanthine or theobromine was added to the reaction solution instead of paraxanthine. As a result of measurement of enzyme activity, production of theobromine was observed in the reaction solution to which 7-methyl xanthine was added instead of paraxanthine, while production of caffeine was observed in the reaction solution to which theobromine was added instead of paraxanthine. Regarding these results, it was revealed that the enzyme isolated in the above procedure had three different N-methyl transferase activities using two other substrates in addition to paraxanthine, respectively.

Example 10

Suppression of Caffeine Synthesis according to an Antisense Method

[0096] A recombinant vector carrying an antisense N-methyl transferase gene was constructed by the following procedure:

[0097] DNA fragments were amplified by PCR using the total length of the isolated N-methyl transferase gene as used in Example 9 as the template and the primers having the nucleotide sequences of ID SEQ NOs: 20 and 21, respectively. The ends of the DNA fragments thus amplified were changed into the blunt ends by BKL kit (TAKARA) to obtain the blunt-ended PCR amplified fragments.

[0098] Separately, pBI vector (Clontech), to which a hygromycin resistance gene connected, was cut with XbaI and SacI to remove the β-glucuronidase gene and the ends of the linear vector thus obtained was changed into the blunt ends.

[0099] The blunt-ended linear vector was ligated with the blunt-ended PCR amplified fragments to obtain recombinant vectors by Ligation Kit (TAKARA), and then the vector carrying the desired N-methyl transferase gene which was inserted in the reverse direction at the operable location on the downstream side of the CaMV35S promoter in the pBI vector was selected from the reaction products by sequencing. Thus, the desired recombinant vector, into which the antisense N-methyl transferase gene was inserted, was obtained and used the following transformation:

[0100] Regarding the conventional methods of biological synthesis of caffeine using coffee tissue culture, there

have been many reports such as Planta, 108, 339 (1972), Plant Cell Reports, 2, 109 (1983). Coffee callus was induced from a coffee shoot apex or a young leaf according to the conventional methods. The recombinant vector was introduced into the callus thus obtained by the particle gun method. Alternatively, protoplasts of the callus was prepared by the conventional methods and the recombinant vector was introduced into the protoplasts by electroporation. After the introduction, cells having the marker resistance were selected. The cells thus selected were cultured under light condition and the enzyme activity of the transformed cells was measured according to the method as described in Example 9. As a result, it was revealed that the caffeine production by the transformed cells into which the antisense N-methyl transferase gene was introduced was significantly reduced in comparison with that of the normal cells into which the antisense N-methyl transferase gene was not introduced.

[0101] Redifferentiation of the transformed coffee cells were conducted to obtain a young coffee plant, whereby the redifferentiation was carried out by a conventional methods described in the reports including Z. Pflanzenphysiol. Bd., 81, 395 (1977); and Plant Cell, Tissue and Organ Culture, 8, 243 (1987). The activity of each of the enzymes from the leaves of the young coffee plant was measured. As a result, it was revealed that caffeine production of the young coffee plant, into which the antisense N-methyl transferase was introduced, was significantly reduced in comparison with that of the young coffee plant, into which the antisense N-methyl transferase gene was not introduced.

[0102] The sequences defined by SEQ ID Numbers are as follows:

SEQ ID NO: 1

Phe Met Asn Arg Gly Glu Gly Glu Ser Ser Tyr Ala Gln Asn Ser
Ser Phe Thr Gln Gln Val Ala Ser Met Ala Gln Pro Ala Leu Glu

Asn Ala Val Glu Thr Leu Phe Ser Arg Asp Phe His Leu Gln Ala
 5 Leu Asn Ala Ala Asp Leu Gly Cys Ala Ala Gly Pro Asn Thr Phe
 Ala Val Ile Ser Thr Ile Lys Arg Met Met Glu Lys Lys Cys Arg
 10 Glu Leu Asn Cys Gln Thr Leu Glu Leu Gln Val Tyr Leu Asn Asp
 Leu Phe Gly Asn Asp Phe Asn Thr Leu Phe Lys Gly Leu Ser Ser
 Glu Val Ile Gly Asn Lys Cys Glu Glu Val Pro Cys Tyr Val Met
 15 Gly Val Pro Gly Ser Phe His Gly Arg Leu Phe Pro Arg Asn Ser
 Leu His Leu Val His Ser Ser Tyr Ser Val His Trp Leu Thr Gln
 Ala Pro Lys Gly Leu Thr Ser Arg Glu Gly Leu Ala Leu Asn Lys
 20 Gly Lys Ile Tyr Ile Ser Lys Thr Ser Pro Pro Val Val Arg Glu
 Ala Tyr Leu Ser Gln Phe His Glu Asp Phe Thr Met Phe Leu Asn
 Ala Arg Ser Gln Glu Val Val Pro Asn Gly Cys Met Val Leu Ile
 25 Leu Arg Gly Arg Gln Cys Ser Asp Pro Ser Asp Met Gln Ser
 Cys Phe Thr Trp Glu Leu Leu Ala Met Ala Ile Ala Glu Leu Val
 Ser Gln Gly Leu Ile Asp Glu Asp Lys Leu Asp Thr Phe Asn Ile
 30 Pro Ser Tyr Phe Ala Ser Leu Glu Glu Val Lys Asp Ile Val Glu
 Arg Asp Gly Ser Phe Thr Ile Asp His Ile Glu Gly Phe Asp Leu
 35 Asp Ser Val Glu Met Gln Glu Asn Asp Lys Trp Val Arg Gly Glu
 Lys Phe Thr Lys Val Val Arg Ala Phe Thr Glu Pro Ile Ile Ser
 Asn Gln Phe Gly Pro Glu Ile Met Asp Lys Leu Tyr Asp Lys Phe
 40 Thr His Ile Val Val Ser Asp Leu Glu Ala Lys Leu Pro Lys Thr
 Thr Ser Ile Ile Leu Val Leu Ser Lys Ile Asp Gly

SEQ ID NO: 2

tgatatcact gctgtggcag ctggcctctt tgctataaaa attacttttc
 tgacgaggca tggagctagc tactgcgggg aaggtgaacg aagtgttgtt
 50 catgaacagg ggggaaggag aaagtagtta tgcacaaaac tcttctttca
 cgcaacaagt ggcctcaatg gcacagccag cgctagaaaa tgcagttgaa

actctcttct ccagagattt ccaccttcaa gctcttaacg cagcggactt
gggttggtgca gcgggtccaa acacattcgc agtgatttct acgatcaaga
5 gaatgatgga aaagaaatgc agggaattga attgccaaac actggaactt
caggtttact tgaatgatct ttttggaat gatttcaata ccctcttcaa
10 aggcctgtcg tctgaggtta ttggtacaa atgtgaggaa gttccgtgtt
atgtgatggg agtaccgggg tctttccatg gccggctttt tctcgtaac
agcttacatt tagttcattc ctcttacagt gttcattggc ttactcaggc
15 accaaaagga ctcacaagca gagaaggctt ggcattaaac aaggggaaga
ttacatatc aaagacaagc cctcctgttg taagagaagc ctacttatct
caatttcatg aagatttcac aatgtttctc aatgctagat cccaagaggt
20 ggttccaaat ggttgatgg tgttgatact tcgtggtagg caatgttctg
atccttcaga catgcagagc tgctttactt ggaactatt agctatggcc
25 attgctgaat tggtttcaca gggattgata gatgaagata aattagacac
cttcaatata cccagctatt ttgcatcact tgaggaagtg aaagatatag
30 tggagagggg cggatcattc acaattgatc atatagaggg gtttgatctt
gatagcgtag aaatgcagga gaatgataaa tgggttagag gggaaaagtt
taccaaggtt gtcaggccct tcacagagcc tataatttca aaccagtttg
35 gacctgaaat catggacaaa ctatatgaca aattcactca cattgtagtt
tcagatttgg aagcaaagct accgaagacc acaagtatca tctagtgtc
40 ttccaagatt gatggatagt tttttagtgt tgtgaaataa actgttgtcc
ctatcacata tatgccacta gagggttggt ccaatgtatt gcacaagaag
45 atttgagagg ggtcaaatat agaaagcatt ttgctcttgt gtggagagag
aatgttttct tgatttaaatt ctgtgatacc caaatcgtaa tgttggaag
aaatgagaag ttgaacatga aattttaaaa aaaaaaaaaa aaaaaaaaaa
50 aaaaaaaatt cctgcggccg cgaattc

SEQ ID NO: 3

ugauaucacu gcuguggcag cuggccucuu ugcuaaaaa auuacuuuuc
 5 ugacgaggca uggagcuagc uacugcggg aaggugaacg aaguguuguu
 caugaacagg ggggaaggag aaaguaguua ugcacaaaac ucuucuuuca
 cgcaacaagu ggccucaaug gcacagccag cgcuaaaaa ugcaguugaa
 10 acucucuucu ccagagauuu ccaccuucua gcucuuaacg cagcggacuu
 gggguugugca gcggguccaa acacauucgc agugauuucu acgaucaaga
 gaaugaugga aaagaaugc agggauuga auugccaaac acuggaacuu
 15 cagguuuacu ugaauaucu uuuuggaaau gauucaaaua cccucuucua
 aggccugucg ucugagguua uugguaacaa augugaggaa guuccguguu
 20 augugauggg aguaccgggg ucuuuccaug gccggcuuuu uccucguaac
 agcuuacauu uaguucauuc cucuuacagu guucauuggc uuacucaggc
 accaaaagga cucacaagca gagaaggcuu ggcauuaaac aaggggaaga
 25 uuuacauauc aaagacaagc ccuccuguug uaagagaagc cuacuuaucu
 caauuucaug aagauuucac aauguuucuc aaugcuagau cccaagaggu
 gguuccaaau gguuguauug uguugauacu ucgugguagg caauguucug
 30 auccuucaga caugcagagc ugcuuuacuu gggacuauu agcuauggcc
 auugcugaau ugguuucaca gggauugaua gaugaagaua aaauagacac
 cuucaauua cccagcuauu uugcaucacu ugaggaagug aaagauauag
 35 uggagaggga cggaucuuuc acaauugauc auauagaggg guuugaucuu
 gauagcguag aaugcagga gaugauaaa uggguuagag gggaaaaguu
 40 uaccaagguu gucagggccu ucacagagcc uauaauuua aaccaguuug
 gaccugaaau cauggacaaa cuauaugaca aaucacuca cauuguaguu
 ucagauuugg aagcaaagcu accgaagacc acaaguauca uccuagugcu
 45 uuccaagauu gauggauagu uuuuuagugu ugugaaauaa acuguugucc
 cuaucacaua uaugccacua gagggugug ccauguauu gcacaagaag
 50 auuugagagg ggucaaauau agaaagcauu uugcucuugu guggagagag
 aauguuuucu ugauuuuuu cugugauacc caaauuguaa uguugggaag
 55

aaaugagaag uugaacauga aaauuuuuuuuu aaaaaaaaaa aaaaaaaaaa

5 aaaaaaaaauu ccugcgggccg cgaauuc

SEQ ID NO: 4

Phe Met Asn Arg Gly Glu Xaa Glu Ser Ser Tyr Ala Gln Asn Ser

10 Gln Phe Thr Gln Val

SEQ ID NO: 5

ttYatgaaYM gIggIgaRg

15

SEQ ID NO: 6

caaaagggtc agtgctgca

20

SEQ ID NO: 7

atgaccatga ttacgcc

SEQ ID NO: 8

25

gccgggtacct ttctggggcc

SEQ ID NO: 9

ccgctgcgtt aagagcttga ag

30

SEQ ID NO: 10

gccaaacact ggaacttcag g

35

SEQ ID NO: 11

ccattgaggc cacttggttg gtg

SEQ ID NO: 12

40

ggcctgtcgt ctgaggttat tg

SEQ ID NO: 13

cagcaatggc catagctaag ag

45

SEQ ID NO: 14

ccgctgcgtt aagagcttga ag

50

SEQ ID NO: 15

gccaaacact ggaacttcag g

55

SEQ ID NO: 16

ccattgaggc cacttggttg .gtg

SEQ ID NO: 17

ggcctgtcgt ctgaggttat tg

SEQ ID NO: 18

gccatggttt acgcgca

SEQ ID NO: 19

cggccatgga aagaccccgg

SEQ ID NO: 20

tgatatcact gctgtggcag c

SEQ ID NO: 21

aaaatttcac gttcaacttc t

aaagtagtta tgcacaaaac tcttcttcca cgcaacaagt ggccctcaatg gcacagccag 180
 cgttagaaaa tgcagtagaa acitcttctt ccagagattt ccaccitcaa gctcttaacg 240
 cagcggactt ggggttgtaga ggggttccaa acacattcgc agtgatttct acgatcaaga 300
 gaalgaigga aaagaaatgc agggaaatga attgccaaac acttggaactt cagggttact 360
 tgaalgaict ttttggaaaat gatttcaata cccitctcaa agggcgtgctg tctgaggtaa 420
 ttggtaacaa atgtgaggaa gticcggtgtt atgtgatggg agtaccgggg tctttccatg 480
 gccggctttt tcttcgtaac agcttacctt tagttacatc cttttacatg gticattggc 540
 ttacitaggc accaaaagga ctcaacaagca gagaaggctt ggcatlaaac aagggaaga 600
 ttacataatc aaagacaagc ccttctgttg taagagaagc ctacttactt caattitacg 660
 aagatttcaac aatgtttctc aatgttagat cccaagaggt ggttccaaat ggtttagatg 720
 tgttagatct tctgttagg caatgttctg atcttccaga catgcagagc tcttttactt 780
 gggaactatt agctatggcc atgtctgaat tggtttccaa gggatigata galgaagata 840
 aatlagacac cttaaatata cccagctatt ttgcatcact ttaggaagtg aaagatatag 900
 tggagaggga cggatcattc acaatitac atatagaggg gtttagatct galagcgtag 960
 aaatgcagga gaalgaataa tgggttagag gggaaaagt taccaaggt gtcagggctt 1020
 tcacagagcc tataatttca aaccagtttg gaccitgaat catggacaaa ctatatgaca 1080
 aatcacitca catgttagtt tcaagatttg aagcaaatgt accgaagacc acaagiatca 1140
 tcttagtctt tttcaagatt gatggaatgt ttttttaggt tgtgaaataa acigtgttcc 1200
 ctatcacata tatgccacta gagggttctg ccaatgtatt gcacaagaag atttgagagg 1260
 ggtcaaatat agaaagcatt ttgtcttctg tggagagag aatgtttctt tgaattataa 1320
 ctgtgatacc caaatctgaa tgttgggaag aaatgagaag ttaacatga aattttaaaa 1380
 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa cctgcggccg cgaattc 1427
 <210> 3
 <211> 1427
 <212> RNA
 <213> Camellia sinensis
 <400> 3
 ugauaucacu gcuguggcag cuggccucuu ugcuauaaaa auuacuuuuc ugacgaggca 60
 uggagcuagc uacugcgggg aaggugaacg aaguguuguu caugaacagg ggggaaggag 120
 aaaguaguuu ugcacaaaac ucuucuuuca cgcaacaagu ggccucaaug gcacagccag 180
 cgcuagaaaa ugcaguugaa acucucucuu ccagagauuu ccaccuucaa gcucuuuacg 240
 cagcggacuu gggguugcga gggguccaa acacauucgc agugauuucu acgaucaaga 300
 gaauaugga aaagaaugc agggaauga auugccaaac acuggaacuu cagguuuacu 360
 ugaauaugcu uuuggaaau gauuucuuu cccucuucaa agggcugucg ucugagguua 420
 uggguaacaa augugaggaa guuccguguu augugauggg aguaccgggg ucuuuuccag 480
 gccggcuuuu uccucguaac agcuuacuu uaguucuuu cucuuacagu guucauuggc 540
 uuacucaggc accaaaagga cucacaagca gagaaggcuu ggcauuuac aaggggaaga 600
 uuucacuuuc aaagacaagc ccuccguug uaagagaagc cuacuuuacu cauuuucag 660
 aagauuucac aauguuucuc aaugcuagau cccaagaggu gguuccaaa gguuguaugg 720
 uguugauacu ucuugguagg caauguucg auccuucaga caugcagagc ugcuuuacu 780
 gggaacuuu agcuauaggc auugcugaau ugguuucaca gggauugaua gaugaagava 840
 aaauagacac cuucaauuaa cccagcuauu uugcaucacu ugaggaagug aaagauuag 900
 uggagaggga cggaucauuc acaauugau auauagagg guuugaucuu gauagcguag 960
 aaugcagga gaauugauaa uggguuagag gggaaaaguu uaccaagguu gucagggccu 1020
 ucacagagcc uauauuuuca aaccaguug gaccugaaau cauggacaaa cuauaugaca 1080
 aaucacuca cauuguaugu ucagauuugg aagcaaaagc accgaagacc acaaguuaca 1140
 ucuuagugcu uucaagauu gauggauagu uuuuuagugu ugugaaauaa acuguugucc 1200
 cuaucacua uaugccacua gaggguugug ccaauguauu gcacaagaag auuugagagg 1260
 ggucuuuuu agaaagcauu uugcucuugu guggagagag aauguuuucu ugaauuuuuu 1320
 cugugauacc caauucguua uguugggag aaugagaag ugaacauga auuuuuuuuu 1380
 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa ccugcggccg cgaauuc 1427
 <210> 4
 <211> 20
 <212> PRT
 <213> Camellia sinensis
 <400> 4
 Phe Met Asn Arg Gly Glu Xaa Glu Ser Ser Tyr Ala Gln Asn Ser Gln
 5 10 15
 Phe Thr Gln Val
 20
 <210> 5
 <211> 19
 <212> DNA
 <400> 5
 ttatgaaYM gggglaRg 19

5
 <210> 6
 <211> 19
 <212> DNA
 <400> 6
 caaaagggtc agtgcigca 19
 <210> 7
 <211> 17
 <212> DNA
 <400> 7
 atgaccatga ttacgcc 17
 10
 <210> 8
 <211> 20
 <212> DNA
 <400> 8
 gccggtacct ttctggggcc 20
 15
 <210> 9
 <211> 22
 <212> DNA
 <400> 9
 ccgctgcgtt aagagcttga ag 22
 20
 <210> 10
 <211> 21
 <212> DNA
 <400> 10
 gccaaacact ggaacttcag g 21
 25
 <210> 11
 <211> 23
 <212> DNA
 <400> 11
 ccattgaggc cacttgttgc gtg 23
 30
 <210> 12
 <211> 22
 <212> DNA
 <400> 12
 ggccctgtcgt ctgaggttat tg 22
 35
 <210> 13
 <211> 22
 <212> DNA
 <400> 13
 cagcaatggc catagctaat ag 22
 40
 <210> 14
 <211> 22
 <212> DNA
 <400> 14
 ccgctgcgtt aagagcttga ag 22
 45
 <210> 15
 <211> 21
 <212> DNA
 <400> 15
 gccaaacact ggaacttcag g 21
 50
 <210> 16
 <211> 23
 <212> DNA
 <400> 16
 ccattgaggc cacttgttgc gtg 23
 55
 <210> 17
 <211> 22
 <212> DNA
 <400> 17
 ggccctgtcgt ctgaggttat tg 22
 <210> 18
 <211> 17
 <212> DNA
 <400> 18

EP 1 055 727 A2

gccatgggtt acgcga 17

<210> 19

<211> 20

<212> DNA

<400> 19

cggccatgga aagaccccgg 20

<210> 20

<211> 21

<212> DNA

<400> 20

tgataacact gctgggcag c 21

<210> 21

<211> 21

<212> DNA

<400> 21

aaaatttcatt gttaacttc t 21

SEQUENCE LISTING

<110> MITSUI CHEMICALS, INC.

<120> Gene Encoding Caffeine Synthesis System Associated Enzyme
and Use thereof

<150> JP 146358/1999

<151> 1999-05-26

<160> 21

<210> 1

<211> 356

<212> PRT

<213> Camellia sinensis

<400> 1

5 Phe Met Asn Arg Gly Glu Gly Glu Ser Ser Tyr Ala Gln Asn Ser Ser
 15 Phe Thr Gln Gln Val Ala Ser Met Ala Gln Pro Ala Leu Glu Asn Ala
 Val Glu Thr Leu Phe Ser Arg Asp Phe His Leu Gln Ala Leu Asn Ala
 20 Ala Asp Leu Gly Cys Ala Ala Gly Pro Asn Thr Phe Ala Val Ile Ser
 Thr Ile Lys Arg Met Met Glu Lys Lys Cys Arg Glu Leu Asn Cys Gln
 25 Asn Thr Leu Phe Lys Gly Leu Ser Ser Glu Val Ile Gly Asn Lys Cys
 Glu Glu Val Pro Cys Tyr Val Met Gly Val Pro Gly Ser Phe His Gly
 30 Arg Leu Phe Pro Arg Asn Ser Leu His Leu Val His Ser Ser Tyr Ser
 Val His Trp Leu Thr Gln Ala Pro Lys Gly Leu Thr Ser Arg Glu Gly
 35 Val Val Arg Glu Ala Tyr Leu Ser Gln Phe His Glu Asp Phe Thr Met
 Phe Leu Asn Ala Arg Ser Gln Glu Val Val Pro Asn Gly Cys Met Val
 40 Leu Ile Leu Arg Gly Arg Gln Cys Ser Asp Pro Ser Asp Met Gln Ser
 Cys Phe Thr Trp Glu Leu Leu Ala Met Ala Ile Ala Glu Leu Val Ser
 45 Tyr Phe Ala Ser Leu Glu Glu Val Lys Asp Ile Val Glu Arg Asp Gly
 Ser Phe Thr Ile Asp His Ile Glu Gly Phe Asp Leu Asp Ser Val Glu
 50 Met Gln Glu Asn Asp Lys Trp Val Arg Gly Glu Lys Phe Thr Lys Val
 Val Arg Ala Phe Thr Glu Pro Ile Ile Ser Asn Gln Phe Gly Pro Glu
 55 Ile Met Asp Lys Leu Tyr Asp Lys Phe Thr His Ile Val Val Ser Asp

Leu Glu Ala Lys Leu Pro Lys Thr Thr Ser Ile Ile Leu Val Leu Ser

5

10

15

20

25

30

35

40

45

50

55

340 345 350
 Lys Ile Asp Gly
 355
 <210> 2
 <211> 1427
 <212> DNA
 <213> *Camellia sinensis*
 <400> 2
 tgatatcact gctgtggcag ctggcctctt tgctataaaa attacttttc tgacgaggca 60
 tggagctagc tactgcgggg aaggtgaacg aagtgttggt catgaacagg qgggaaggag 120
 aaagtagtta tgcacaaaac tcttctttca cgcaacaagt ggcctcaatg gcacagccag 180
 cgctagaaaa tgcagttgaa actctcttct ccagagattt ccaccttcaa gctcttaacg 240
 cagcggactt ggggttggtgca gcgggtccaa acacattcgc agtgatttct acgatcaaga 300
 gaatgatgga aaagaaatgc aggggaattga attgccaaac actggaaactt caggtttact 360
 tgaatgatct ttttggaat gatttcaata ccctcttcaa aggcctgtcg tctgaggtta 420
 ttggtataca atgtgaggaa gttccgtggt atgtgatggg agtaccgggg tctttccatg 480
 gccggctttt tctcgtgaac agcttacatt tagttcattc ctcttacagt gttcattggc 540
 ttactcaggc accaaaagga ctcaacaagc gagaaggctt ggcattaaac aaggggaaga 600
 ttacatatac aaagacaagc cctcctgttg taagagaagc ctacttatct caatttcatg 660
 aagattttcac aatgtttctc aatgctagat cccaagaggt ggttccaaat ggttgatgg 720
 tgttgatact tctgtgtagg caatgttctg atccttcaga catgcagagc tgctttactt 780
 gggaactatt agctatggcc attgctgaat tggtttcaca gggattgata gatgaagata 840
 aattagacac cttcaatata cccaagctatt ttgcatcact tgagggaagt aaagatatag 900
 tggagaggga cggatcattc acaattgatc atatagaggg gtttgatctt gatagcgtag 960
 aaatgcagga gaatgataaa tgggttagag gggaaaagt taccagggtt gtcaggccct 1020
 tcacagagcc tataatttca aaccagtttg gacctgaaat catggacaaa ctatatgaca 1080
 aattcactca cattgtagtt tcagatttgg aagcaaagct accgaagacc acaagtatca 1140
 tctagtgtct ttccaagatt gatggatagt tttttagtgt tgtgaaataa actggtgtcc 1200
 ctatcacata tatgccacta gaggtttagt ccaatgtatt gcacaagaag atttgagagg 1260
 ggtcaaatat agaaagcatt ttgctcttgt gtggagagag aatgttttct tgatttaaat 1320
 ctgtgatacc caaatcgtaa tgttggaag aaatgagaag ttgaacatga aatttttaaaa 1380
 aaaaaaaaaa aaaaaaaaaa aaaaaaaatt cctgcggccg cgaattc 1427
 <210> 3
 <211> 1427
 <212> RNA
 <213> *Camellia sinensis*
 <400> 3
 ugauaucacu gcuguggcag cuggccucuu ugcuaaaaa auuacuuuuc ugacgaggca 60
 uggagcuagc uacugcgggg aaggugaacg aaguguuguu caugaacagg ggggaaggag 120
 aaaguaguua ugcacaaaac ucuucuuuca cgcaacaagu ggccucaaag gcacagccag 180
 cgcuagaaaa ugcauguuga acucucuuucu ccagagauuu ccaccuucaa gcucuaaacg 240
 cagcggacuu gggguuguca gcggguccaa acacauucgc agugauuuucu acgaucaaga 300
 gaaugaugga aaagaaaugc agggaaauuga auugccaaac acuggaacuu cagguuuacu 360
 ugaaugaucu uuugggaaau gauuucaaua ccucuucaa aggcugucg ucugagguua 420
 uugguaacaa augugaggaa guuccguguu augugauggg aguaccgggg ucuuuccaug 480
 gccggcuuuu uccucguaac agcuuacauu uaguucuuu cucuuacagu guucauuggc 540
 uuacucaggc accaaaagga cucacaagca gagaaggcuu ggcauuuaac aaggggaaga 600
 uuuaucuuuc aaagacaagc ccuccuguug uaagagaagc cuacuuuacu cauuuucaug 660
 aagauuucac aauguuucuc aaugcuagau cccaagaggu gguuccaaau gguuguauug 720
 uguugauacu ucgugguagg caauguucug auccuucaga caugcagagc ugcuuucuu 780

5
 10
 15
 20
 25
 30
 35
 40
 45
 50
 55

gggaacuaau agcuauaggcc auugcugaau ugguuucaca gggauugaua gaugaagaua 840
 aaauagacac cuucaauaua cccagcuauu ugcaucacu ugaggaagug aaagauauag 900
 uggagaggga cggaucauuc acaauugauc auauagaggg guuugaucuu gauagcguag 960
 aaauagcagga gaaugauaaa ugguuagag gggaaaaguu uaccaaggguu gucagggccu 1020
 ucacagagcc uauaauuuaa aaccaguug gaccugaaau cauggacaaa cuauaugaca 1080

aaucacuca cauuguaguu ucagauuugg aagcaaagcu accgaagacc acaaguauca 1140
 uccuagugcu uuccaagauu gauggauagu uuuuuagugu ugugaaaua acuguugucc 1200
 cuaucacaua uaugccacua gagggguugug ccaauguauu gcacaagaag auuugagagg 1260
 ggucaaaaua agaaagcauu uugcucuugu guggagagag aauguuuucu ugauuuuuuu 1320
 cugugauacc caaaucguaa uguugggaag aaugagaag uugaacauga aauuuuuuuu 1380
 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa ccugcggccg cgaaauuc 1427

<210> 4

<211> 20

<212> PRT

<213> Camellia sinensis

<400> 4

Phe Met Asn Arg Gly Glu Xaa Glu Ser Ser Tyr Ala Gln Asn Ser Gln

5

10

15

Phe Thr Gln Val

20

<210> 5

<211> 19

<212> DNA

<400> 5

ttYatgaaYM gIggIgaRg 19

<210> 6

<211> 19

<212> DNA

<400> 6

caaaaggggtc agtgctgca 19

<210> 7

<211> 17

<212> DNA

<400> 7

atgaccatga ttacgcc 17

<210> 8

<211> 20

<212> DNA

<400> 8

gccggtacct ttctggggcc 20

<210> 9

<211> 22

<212> DNA

<400> 9

ccgctgcgtt aagagcttga ag 22

<210> 10

<211> 21

<212> DNA

<400> 10

gccaaacact ggaacttcag g 21

EP 1 055 727 A2

<210> 11
 <211> 23
 <212> DNA
 <400> 11
 ccattgaggc cacttggtgc gtg 23
 <210> 12
 <211> 22
 <212> DNA
 <400> 12

ggcctgtcgt ctgaggttat tg 22

<210> 13
 <211> 22
 <212> DNA
 <400> 13
 cagcaatggc catagctaag ag 22
 <210> 14
 <211> 22
 <212> DNA
 <400> 14
 ccgctgctgt aagagcttga ag 22

<210> 15
 <211> 21
 <212> DNA
 <400> 15
 gccaaacact ggaacttcag g 21

<210> 16
 <211> 23
 <212> DNA
 <400> 16
 ccattgaggc cacttggtgc gtg 23
 <210> 17
 <211> 22
 <212> DNA
 <400> 17
 ggcctgtcgt ctgaggttat tg 22

<210> 18
 <211> 17
 <212> DNA
 <400> 18
 gccatgggtt acgcga 17

<210> 19
 <211> 20
 <212> DNA
 <400> 19
 cggccatgga aagaccccg 20

<210> 20
 <211> 21
 <212> DNA
 <400> 20
 tgatatcact gctgtggcag c 21
 <210> 21

EP 1 055 727 A2

<211> 21

<212> DNA

<400> 21

aaaatttcac gttcaacttc t 21

5

10

15

20

25

30

35

40

45

50

55

<110> MITSUI CHEMICALS, INC.

5

74

11

2

14

15

20

1

25

		340		345		350	
		Lys Ile Asp Gly					
		355					
5		<210> 2					
		<211> 1427					
		<212> DNA					
		<213> Camellia sinensis					
		<400> 2					
10		tgatatcact gctgtggcag ctggcctctt tgctataaaa attacttttc tgacgaggca 60					
		tgagagctagc tactgcgggg aagggtgaacg aagtgttggt catgaacagg ggggaaggag 120					
		aaagtagtta tgcacaaaac tcttctttca cgcaacaagt ggcctcaatg gcacagccag 180					
		cgctagaaaa tgcagttgaa actctcttct ccagagattt ccaccttcaa gctcttaacg 240					
		cagcggactt gggttgtgca gcgggtccaa acacattcgc agtgatttct acgatcaaga 300					
		gaatgatgga aaagaaatgc aggggaattga attgccaaac actggaactt caggtttact 360					
15		tgaatgatct ttttggaat gatttcaata ccctcttcaa aggcctgtcg tctgaggtta 420					
		ttggtaacaa atgtgaggaa gttccgtggt atgtgatggg agtacggggg tctttccatg 480					
		gccggctttt tcctcgtaac agcttacatt tagttcattc ctcttacagt gttcattggc 540					
		ttactcaggc accaaaagga ctcacaagca gagaaggctt ggcattaaac aagggggaaga 600					
		tttaccatctc aaagacaagc cctcctgttg taagagaagc ctacttatct caatttccatg 660					
		aagatttcac aatgtttctc aatgctagat cccaagagggt ggttccaaat ggttgtatgg 720					
20		tggtgatact tcgtggtagg caatgttctg atccttcaga catgcagagc tgctttactt 780					
		gggaactatt agctatggcc attgctgaat tggtttcaca gggattgata gatgaagata 840					
		aattagacac ctccaatata cccagctatt ttgcatcact tgaggaaagt aaagatatag 900					
		tggagaggga cggatcattc acaattgatc atatagagggt gtttgatctt gatagcgtag 960					
		aaatgcagga gaatgataaa tgggttagag gggaaaagtt taccaagggt gtcagggcct 1020					
		tcacagagcc tataatattca aaccagtttg gacctgaaat catggacaaa ctatatgaca 1080					
25		aattcactca cattgtagtt tcagatttgg aagcaaaagt accgaagacc acaagtatca 1140					
		tcctagtgtt ttccaagatt gatggatagt ttttttagtgt tgtgaaataa actgttgtcc 1200					
		ctatcacata tatgccacta gaggggtgtg ccaatgtatt gcacaagaag atttgagagg 1260					
		ggtcaaatat agaaagcatt ttgctcttgt gtggagagag aatgttttct tgatttaaat 1320					
		ctgtgatacc caaatcgtaa tgttgggaag aaatgagaag ttgaacatga aattttaaaa 1380					
		aaaaaaaaa aaaaaaaaaa aaaaaaaatt cctgcggccg cgaattc 1427					
30		<210> 3					
		<211> 1427					
		<212> RNA					
		<213> Camellia sinensis					
		<400> 3					
35		ugauaucacu gcuguggcag cuggccucuu ugcuaaaaaa auuacuuuuc ugacgaggca 60					
		uggagcuagc uacugcgggg aaggugaacg aaguguuguu caugaacagg ggggaaggag 120					
		aaaguaguua ugcacaaaac ucuuuuuua cgcaacaagu ggcucuuaug gcacagccag 180					
		cgcuagaaaa ugcaguugaa acucucuuc cagagauuu ccaccuucaa gcucuuaacg 240					
		cagcggacu ggguugugca gcggguccaa acacauucgc agugauuuc acgaucaaga 300					
		gaauaugga aaagaaaugc agggaaauuga auugccaaac acuggaacu cagguuuacu 360					
40		ugaaugaucu uuugggaaa gaauucaaua ccucuucaa aggccugucg ucugagguua 420					
		uugguuacaa augugaggaa guuccguguu augugauggg aguaccgggg ucuuuccaug 480					
		gccggcuuuu uccucguaac agcuuacauu uaguucauuc cucuuacagu guucauuggc 540					
		uuacucaggc accaaaagga cucacaagca gagaaggcuu ggcauuaaac aagggggaaga 600					
		uuuacauauc aaagacaagc ccuccuguu uaaagagaagc cuacuuuacu caauuucaug 660					
		aagauuucac aauguuucuc aaugcuagau cccaagaggu gguuccaaa gguuguauug 720					
45		uguugauacu ucgugguagg caauguucug auccuucaga caugcagagc ugcuuuacu 780					
		gggaacuauu agcuauggcc auugcugaau ugguuucaca gggauugaua gaugaagaua 840					
		aaauagacac cuucaauuaa cccagcuauu uuugcaucacu ugaggaagug aaagauauag 900					
		uggagaggga cggaucuuuc acaauugauc auauagaggg guuugaucuu gauagcguag 960					
		aaaugcagga gaaugauaaa uggguuagag ggggaaaagu uaccaagguu gucagggccu 1020					
50		ucacagagcc uauauuuuca aaccaguug gaccugaaau cauggacaaa cuauaugaca 1080					

aaucacuca cauguaguuu ucagauuugg aagcaaagcu accgaagacc acaagauca 1140
 uccuagugcu uuccaagauu gauggauagu uuuuuagugu ugugaaaaua acuguugucc 1200
 cuaucacaua uauGCCacua gagggauugug ccaauguauu gcacaagaag auuugagagg 1260
 ggucaaaauu agaaagcauu uugcucuugu guggagagag aauguuuucu ugauuuuuuu 1320
 cugugauacc caaaucguaa uguugggaag aaugagagaag uugaacauga aaauuuuuuu 1380
 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa ccugcggccg cgaaauuc 1420

<210> 4

<211> 20

<212> PRT

<213> Camellia sinensis

<400> 4

Phe Met Asn Arg Gly Glu Xaa Glu Ser Ser Tyr Ala Gln Asn Ser Gln

Phe Thr Gln Val

20

<210> 5

<211> 19

<212> DNA

<400> 5

ttYatgaayM gIggIgaRg 19

<210> 6

<211> 19

<212> DNA

<400> 6

caaaagggtc agtgctgca 19

<210> 7

<211> 17

<212> DNA

<400> 7

atgaccatga ttacgcc 17

<210> 8

<211> 20

<212> DNA

<400> 8

gccggtacct ttctggggcc 20

<210> 9

<211> 22

<212> DNA

<400> 9

ccgctgcgtt aagagcttga ag 22

<210> 10

<211> 21

<212> DNA

<400> 10

gccaaacact ggaacttcag g 21

<210> 11

<211> 23

<212> DNA

<400> 11

ccattgaggc cacttggtgc gtc 23

<210> 12

<211> 22

<212> DNA

<400> 12

5 ggcctgtcgt ctgaggttat tg 22
 <210> 13
 <211> 22
 <212> DNA
 <400> 13
 10 cagcaatggc catagctaata ag 22
 <210> 14
 <211> 22
 <212> DNA
 <400> 14
 15 ccgctgcgtt aagagcttga ag 22
 <210> 15
 <211> 21
 <212> DNA
 <400> 15
 20 gccaaacact ggaacttcag g 21
 <210> 16
 <211> 23
 <212> DNA
 <400> 16
 25 ccattgaggc cacttggtgc gtg 23
 <210> 17
 <211> 22
 <212> DNA
 <400> 17
 30 ggcctgtcgt ctgaggttat tg 22
 <210> 18
 <211> 17
 <212> DNA
 <400> 18
 35 gccatggttt acgcgca 17
 <210> 19
 <211> 20
 <212> DNA
 <400> 19
 40 cggccatgga aagaccccg 20
 <210> 20
 <211> 21
 <212> DNA
 <400> 20
 45 tgatatcact gctgtggcag c 21
 <210> 21
 <211> 21
 <212> DNA
 <400> 21
 50 aaaatttcac gttcaacttc t 21

Claims

55

1. A DNA molecule comprising any of the following nucleotide sequences:

(a) a nucleotide sequence encoding N-methyl transferase that is a polypeptide having an amino acid sequence

of SEQ ID NO: 1 of the sequence listing and having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase, and paraxanthine N3 methyl transferase; and

(b) a modified nucleotide sequence obtained by carrying out nucleotide replacement, deletion, or insertion in said nucleotide sequence (a) within a range where a polypeptide encoded by said nucleotide sequence (a) can maintain said enzyme activities.

2. The DNA molecule as claimed in claim 1, wherein said nucleotide sequence (a) and said modified nucleotide sequence (b) can be hybridized under stringent conditions.

3. The DNA molecule as claimed in claim 1 or 2, wherein said nucleotide sequence (a) consists of a nucleotide sequence of SEQ ID NO: 2 of the sequence listing.

4. An RNA molecule comprising any of the following nucleotide sequences:

(a) a nucleotide sequence encoding N-methyl transferase that is a polypeptide having an amino acid sequence of SEQ ID NO: 1 of the sequence listing and having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase, and paraxanthine N3 methyl transferase; and

(b) a modified nucleotide sequence obtained by carrying out nucleotide replacement, deletion, or insertion in said nucleotide sequence (a) within a range where a polypeptide encoded by said nucleotide sequence (a) can maintain said enzyme activities.

5. An RNA molecule as claimed in claim 4, wherein said nucleotide sequence (a) and said modified nucleotide sequence (b) can be hybridized under stringent conditions.

6. The RNA molecule as claimed in claim 4 or 5, wherein said sequence (a) consists of a nucleotide sequence of SEQ ID NO: 3 of the sequence listing.

7. An expression vector comprising the DNA molecule as claimed in any of claims 1 to 3 and a constitution for expressing said N-methyl transferase encoded by the DNA molecule in plant cells.

8. A transformed cell obtained by transforming a host cell, such as a microorganism cell, with the expression vector as claimed in claim 7.

9. A method for producing N-methyl transferase comprising: culturing the transformed cell as claimed in claim 8 or 9, thereby having said enzyme activities.

10. A DNA molecule comprising a nucleotide sequence complementary to all or part of the nucleotide sequence of the DNA molecule as claimed in any of claims 1 to 3, wherein the enzyme activities of the plant cells can be inhibited when introduced into plant cells having said enzyme activities and is expressed.

11. An RNA molecule comprising a nucleotide sequence complementary to all or part of the nucleotide sequence of the RNA molecule as claimed in any of claims 4 to 6, wherein the enzyme activities of the plant cells can be inhibited when introduced into plant cells having said enzyme activities and is expressed.

12. A vector comprising a DNA molecule or an RNA molecule as claimed in any of claims 1 to 6, 10 and 11, and desirable the vector is capable of expressing N-methyl transferase having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase, and paraxanthine N3 methyl transferase in cells of at least one of microorganisms and plants or having a function of inhibiting the expression of the N-methyl transferase.

13. A microorganism wherein the microorganism is transformed with the vector as claimed in claim 12.

14. A plant cell, plant tissue, or plant body wherein the plant cell, plant tissue, or plant body is transformed with the vector as claimed in claim 12, which for instance is introduced by infection.

15. A method for producing a plant secondary metabolite, or a method for modifying a composition of a plant secondary metabolite comprising: using the plant cell, plant tissue, or plant body as claimed in claim 14.

16. A method for producing plant secondary metabolite comprising: culturing the plant cell or plant tissue as claimed

in claim 14, and growing the plant body.

17. A method for modifying a composition of the plant secondary metabolite comprising: culturing the plant cell or plant tissue, or growing plant body as claimed in claim 14.

18. The method as claimed in any of claims 15 to 17, wherein (a) the plant secondary metabolite is at least one or more compounds selected from the group consisting of 7-methyl xanthine, paraxanthine, theobromine, and caffeine; or (b) wherein a transformed plant body is a Camellia plant, a Coffea plant, Cola plant, Ilex plant, Neea plant, Firmiana plant, Paullinia plant, or Therbroma plant body.

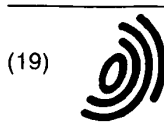
19. An N-methyl transferase having enzyme activities of 7-methyl xanthine N3 methyl transferase, theobromine N1 methyl transferase, and paraxanthine N3 methyl transferase, said N-methyl transferase comprising:

(a) an amino acid sequence of SEQ ID NO: 1 of the sequence listing; or

(b) a modified amino acid sequence obtained by carrying out amino acid replacement, insertion or deletion in the amino acid sequence of SEQ ID NO: 1 of the sequence listing within a range where said enzyme activities are not damaged.

20. The N-methyl transferase as claimed in claim 25, wherein said nucleotide sequence encoding said amino acid sequence (a) and said nucleotide sequence encoding said modified amino acid sequence (b) can hybridize under stringent conditions.

THIS PAGE BLANK (USPTO)



(19)

Eur päisches Patentamt
European Patent Office
Office européen des brevets



(11)

EP 1 055 727 A3

(12)

EUROPEAN PATENT APPLICATION

(88) Date of publication A3:
19.09.2001 Bulletin 2001/38

(51) Int Cl.7: **C12N 15/54**, C12N 15/82,
C12N 15/11, C12N 9/10,
A01H 5/00

(43) Date of publication A2:
29.11.2000 Bulletin 2000/48

(21) Application number: **00304522.6**

(22) Date of filing: **26.05.2000**

(84) Designated Contracting States:
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU
MC NL PT SE**
Designated Extension States:
AL LT LV MK RO SI

- **Ashihara, Hiroshi**
Tokyo (JP)
- **Mizuno, Kouichi**
Ushika-shi, Ibaraki (JP)
- **Fujimura, Tatsuhito**
Tsukuba-shi, Ibaraki (JP)

(30) Priority: **26.05.1999 JP 14635899**

(71) Applicant: **MITSUI CHEMICALS, INC.**
Tokyo (JP)

(74) Representative: **Harvey, David Gareth et al**
Graham Watt & Co.
Riverhead
Sevenoaks Kent TN13 2BN (GB)

(72) Inventors:
• **Mizuno, Misako**
Ushiku-shi, Ibaraki (JP)

(54) Cloning of an N-methyltransferase involved in caffeine biosynthesis

(57) Thanks to the present invention, N-methyl transferase that can be utilized as an industrial, food, or medical enzyme, can be produced efficiently. The present invention makes it possible to modify caffeine biosynthesis metabolism of caffeine productive plants,

plant tissues, or plant cells, for efficiently producing caffeine metabolism based compounds. Furthermore, the caffeine biosynthesis metabolism of caffeine productive plants, plant tissues, or plant cells can be modified, thereby modifying the production rate of a caffeine metabolism based compound group.

EP 1 055 727 A3



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 00 30 4522

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	KATO M ET AL: "CAFFEINE BIOSYNTHESIS IN YOUNG LEAVES OF CAMELLIA SINENSIS: IN VITRO STUDIES ON NU-METHYLTRANSFERASE ACTIVITY INVOLVED IN THE CONVERSION OF XANTHOSINE TO CAFFEINE" PHYSIOLOGIA PLANTARUM, DK, MUNKSGAARD INTERNATIONAL PUBLISHERS, COPENHAGEN, vol. 98, no. 3, 1996, pages 629-636, XP002071639 ISSN: 0031-9317 * the whole document *		C12N15/54 C12N15/82 C12N15/11 C12N9/10 A01H5/00
A	ASHIHARA HIROSHI ET AL: "Biosynthesis and metabolism of caffeine and related purine alkaloids in plants." ADVANCES IN BOTANICAL RESEARCH, vol. 30, 1999, pages 117-205, XP001010585 1999 Academic Press Limited; Academic Press Limited 24-28 Oval Road, London, NW1 7DX, UK; 525 B Street, Suite 1900, San Diego, CA, 92101-4495, USA ISBN: 0-12-005930-4 * see whole document, particularly IV-B *		TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12N A01H
A	WO 98 36053 A (STILES JOHN I; UNIV HAWAII (US); MOISYADI ISTEFO (US); NEUPANE KAB) 20 August 1998 (1998-08-20) * the whole document *		
P, X	KATO MISAKO ET AL: "Purification and characterization of caffeine synthase from tea leaves." PLANT PHYSIOLOGY (ROCKVILLE), vol. 120, no. 2, June 1999 (1999-06), pages 579-586, XP002173207 ISSN: 0032-0889 * the whole document *	19	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 26 July 2001	Examiner Smalt, R
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03/82 (P/C/D)



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 00 30 4522

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
T	<p>KATO, M. ET AL.: "Caffeine synthase gene from tea leaves." NATURE, vol. 406, 31 August 2000 (2000-08-31), pages 956-957, XP002173208 * the whole document *</p> <p>-----</p>		
			<p>TECHNICAL FIELDS SEARCHED (Int.Cl.7)</p>
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 26 July 2001	Examiner Smalt, R
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p>		<p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>	

EPO FORM 1503 03.82 (P4/C01)

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 00 30 4522

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

26-07-2001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9836053 A	20-08-1998	AU 6324798 A	08-09-1998

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82